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(*S*)-*N*-(2,5-Dimethylphenyl)-1-(quinoline-8-ylsulfonyl)pyrrolidine-2-carboxamide as a Small Molecule Inhibitor Probe for the Study of Respiratory Syncytial Virus Infection

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Abstract

A high-throughput, cell-based screen was used to identify chemotypes as inhibitors for human respiratory syncytial virus (*h*RSV). Optimization of a sulfonylpyrrolidine scaffold resulted in compound **5o** that inhibited a virus-induced cytopathic effect in the entry stage of infection (EC_{50} = 2.3 ± 0.8 μM) with marginal cytotoxicity (CC_{50} = 30.9 ± 1.1 μM) and reduced viral titer by 100-fold. Compared to ribavirin, sulfonylpyrrolidine **5o** demonstrated an improved *in vitro* potency and selectivity index.

INTRODUCTION

Human respiratory syncytial virus (*h*RSV) is the most common cause of bronchiolitis and pneumonia among infants and children under one year of age.[1] In the United States there are approximately 125,000 yearly *h*RSV-related hospitalizations, and of those, 500 young children could die due to the infection or its complications each year.[2] The virus is highly contagious and affects those with compromised cardiac, pulmonary (COPD), and immune systems.[3] As such, the elderly are also a highly susceptible population to *h*RSV. Treatment options are limited. Due to infant mortality associated with attempted vaccination, vaccine development is proceeding cautiously.[4,5] Synagis® (Palivizumab), a humanized monoclonal antibody, is a prophylactic, injectable therapeutic used only with high risk pediatric patients.[6] Ribavirin, a nucleoside antimetabolite, is approved for acute infection[7,8] and infected, immunocompromised patients,[9] but has a long half-life and accumulates in erythrocytes, thus requiring regeneration of the affected red blood cells to eliminate the

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Supporting Information. Assay details and experimental characterization for select compounds. This material is available free of charge via the internet at http://pubs.acs.org.

drug. These issues, coupled with embryocidal and teratogenic effects constitute severe toxicological liabilities that limit the use of ribavirin, especially in infants,[7] around administering pregnant medical personnel, and in treated partners of pregnant women (Figure 1).

To address the absence of clinically relevant and safe *h*RSV therapies, many investigators have pursued a target-based drug design approach. Ribavirin 5′-monophosphate resembles GMP and can decrease cellular GTP pools due to the inhibition of the enzyme inosine monophosphate dehydrogenase (IMPDH).[10,11] Nevertheless, this decrease does not completely account for the observed antiviral activity as inhibitory effects have been noted on RNA capping[12] and direct inhibition of viral polymerase activity for influenza viruses.[13] Literature disclosed IMPDH inhibitors have reported *in vitro* therapeutic indices that are not competitive with ribavirin.[14] Several other inhibitors that target the fusion protein,[15–20] ribonucleoprotein (RNP) complex,[21] guanylylation events,[22] and N-protein[23] have been discovered. Of these, some demonstrate potency and limited toxicity in animal models.[24–26] However, due to formulation for oral bioavailability,[19] strategic reasons,[27] and observed loss of activity *in vivo*,[28] many have not progressed to the clinic to combat *h*RSV disease.

In an effort to identify lead compounds acting through new mechanisms, we developed, optimized and validated a high-throughput cell-based screen that measures the respiratory syncytial virus-induced cytopathic effect (CPE) in HEp-2 cells (unpublished results). CPE was measured using the Cell Titer-Glo™ viability assay in which the luminescent signal generated is directly proportional to the amount of cellular ATP present which is also proportional to the number of metabolically active cells. A total of 313,816 compounds from the Molecular Libraries Small Molecule Repository (MLSMR) were screened in single dose against *h*RSV (strain Long) at a concentration of 10 μM. Hits (2,465 compounds) were evaluated for their antiviral activity and cell toxicity in dose response experiments, and 409 compounds produced a protective effect of at least 50% CPE inhibition. Based on potency, selectivity and chemical tractability, 51 hits were selected for verification in an *in vitro* titer reduction assay to assess their effect on the production of infectious progeny virus. Many chemotypes of interest emerged, including several compounds containing a sulfonylpyrrolidine moiety. Hit compound **2** displayed a CPE EC_{50} of 5.0 μM, a CC_{50} (HEp-2 cellular cytotoxicity) of 31.5 μM, and a selectivity index (CC_{50}/EC_{50}) of 6.3 (Figure 2). Profile improvements were explored by modulating various structural elements (shaded regions, Figure 2).

CHEMISTRY

Analogs were prepared by treating substituted sulfonyl chlorides **3** with proline or another suitable amino acid to generate sulfonamide carboxylates **4**. Coupling of the acids to various amines via traditional methods afforded the desired products **5** (Scheme 1).

RESULTS AND DISCUSSION

Attention was first placed on the proline unit between the aryl sulfone and aniline groups. The hit compound **2** was tested as a racemic mixture; therefore, each enantiomer was individually prepared from *L*- or *D*-proline to determine the more pharmacologically active constituent. The *S*-enantiomer, derived from *L*-proline, was found to be the more active component of the racemic mixture, delivering a selectivity index of 11.8 when cytotoxicity was accounted for (entry 4, Table 1). This compound showed a 1 log reduction in virus titer in a plaque reduction assay. Acyclic variants of the linker region, including those that probed the methylation of the amide nitrogen, were found to have EC_{50} values > 50.0 μM.

Several variants of the quinoline moiety were pursued but offered no benefit in potency. Replacing the nitrogen atom with –CH- afforded an inactive analog (EC_{50} > 50.0 μM), as did migrating the nitrogen to alternate positions of the quinoline substructure. The replacement of the quinoline with a 4-linked-benzooxadiazole, a phenyl ring or a simple methyl group was also not advantageous. Substitution of the sulfonyl group (SO_2) for a carbonyl functionality or its replacement with –CH_2- resulted in complete loss of potency (EC_{50} > 50.0 μM, see supporting information, Tables S1 and S2, respectively). Focus was then shifted to the modification of the 2,4-dimethylanilide. Following the results noted above, all analogs in this study were prepared as the *S*-enantiomer (Table 2). Simplifying the 2,4-dimethylphenyl substitution pattern down to monomethyl substituted phenyl derivatives revealed that the observed potency was not due to the presence of one group alone (entries 3–5). Increased steric bulk at C2 marginally improved potency vs. monomethyl substitution at the same position (*cf.* entries 8 to 7). Mimicking the 2,4-substitution pattern with chlorine atoms in place of methyl groups resulted in complete loss of potency (**5n**, entry 11). As the 2-alkyl substituent appeared to be necessary in combination with other substituents to preserve potency, this dynamic was explored further to reveal that the 2,5-dimethylphenyl moiety of analog **5o** was slightly more beneficial in terms of potency and maintained the cytotoxicity threshold (entry 12, EC_{50} = 2.3 μM, CC_{50} = 30.9 μM). For select compounds assessed for aqueous solubility, no effect was observed on CPE potency. Solubility and stability were determined for **5o**, revealing an acceptable solubility measurement of 92.7 μg/mL in PBS buffer and stability of 95.4% (unchanged parent remaining) after 48 h in 50% PBS/50% acetonitrile.[29]

To probe the mechanism of action of the sulfonylpyrrolidines, the window of inhibitory activity in the cell-based assay was refined. Potency of compounds over time following infection was examined to ascertain early (entry) or late (replication) antiviral activity in the virus life cycle.[30]

In the time of addition study, HEp-2 cells were infected with *h*RSV strain Long at an MOI of 3.0 at time point 0 and incubated for 6 days and test compounds, **5b**, **5t**, **5o**, or ribavirin were added to plates at −1, 0, 1, 2, 3, 5, 7, 21 and 24 h post infection (p.i.). CPE was assessed using Cell-Titer Glo as an endpoint reagent. Controls without test compound included HEp-2 cells with no *h*RSV exposure (cell control) and *h*RSV-infected cells (virus control). To evaluate cellular toxicity attributed to test compound alone, uninfected HEp-2 cells were treated with **5b**, **5t**, **5o**, or ribavirin at 25 μM concentration at time point 0, and cell viability was assessed after 144 hours.

There was less than 1% cell viability for the *h*RSV-infected cells without addition of any test compound or ribavirin. Uninfected cells treated with ribavirin, **5t** or **5o** displayed 90%, 88% and 95% cell viability, respectively, indicating low cellular toxicity inherent to these compounds. However, uninfected cells treated with **5b** exhibited only 46% cellular viability, suggesting moderate toxicity due to the test compound alone.[31] Ribavirin treatment protected cells from *h*RSV induced CPE for up to 7 h p.i., indicating that it targets the period of infection during which viral replication is in progress. Compound **5o** protected cells from *h*RSV induced CPE (> 50%) from 1 – 3 h p.i., and at 24 h p.i. cell viability was only 26%. (Figure 3).

Two analogs of similar profile to **5o**, **5b** and **5t**, demonstrated a decrease in efficacy when added at each time point from 0–5 hours p.i. This profile could be due to the inhibition of one or more early virus life cycle steps (entry, post-entry, or early-stage infection processes), a hypothesis that is supported by an inability of these compounds to affect processes later in the viral replication cycle. This data lead us to conclude that **5o** was inhibiting early infection events, characterized by viral attachment, uptake, fusion or initial transcription.

The sulfonylpyrrolidine scaffold analogues were evaluated for their ability to reduce the amount of infectious virus produced in cell culture. These measurements of compound-mediated viral titer reduction were used to complement the cytoprotection assay results. A standard plaque-reduction assay was used as a secondary assay to determine the ability of this class of compounds to reduce the amount of infectious virus produced in HEp-2 cells.[32] Cells were infected with *h*RSV in the presence of 25 μM test compound (**5b**, **5o**, or **5t**). Compounds **5b** and **5t** each showed 1 log reduction in virus titer, or 10-fold, as compared to ribavirin which reduced viral titer by 2.5 log units, or ~300-fold. Analog **5o** showed a 100-fold, or 2 log, reduction. Improvements in cell protection against *h*RSV did not translate to significant improvement in the plaque assay as was seen with ribavirin. Consequently, the titer reduction assay was not used to drive SAR efforts.

CONCLUSION

In summary, the HTS and chemistry optimization efforts produced a series of enantiomerically pure, sulfonylpyrrolidine-based compounds that are effective *in vitro* inhibitors of *h*RSV in the low micromolar range. Many of these compounds were shown to reduce the *in vitro* viral titer by 100-fold. The therapeutic index for the series was maximized at 13.4-fold and is an issue for further refinement preceding *in vivo* assessment.

EXPERIMENTAL SECTION

Chemistry

All final compounds were confirmed to be of >95% purity based on HPLC analysis. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AM 400 spectrometer (operating at 400 and 101 MHz respectively) or a Bruker AVIII spectrometer (operating at 500 and 126 MHz respectively) in $CDCl_3$ with 0.03% TMS as an internal standard or DMSO-d_6. The chemical shifts (δ) reported are given in parts per million (ppm) and the coupling constants (*J*) are in Hertz (Hz). The spin multiplicities are reported as s = singlet, bs = broad singlet, d = doublet, t = triplet, q = quartet, dd = doublet of doublet and m = multiplet. The LCMS analysis was performed on an Agilent 1200 RRL chromatograph with photodiode array UV detection and an Agilent 6224 TOF mass spectrometer. The chromatographic method utilized the following parameters: a Waters Acquity BEH C-18 2.1 × 50mm, 1.7 μm column; UV detection wavelength = 214 nm; flow rate = 0.4ml/min; gradient = 5 – 100% acetonitrile over 3 minutes with a hold of 0.8 minutes at 100% acetonitrile; the aqueous mobile phase contained 0.15% ammonium hydroxide (v/v). The mass spectrometer utilized the following parameters: an Agilent multimode source which simultaneously acquires ESI+/APCI+; a reference mass solution consisting of purine and hexakis(1H, 1H, 3H-tetrafluoropropoxy) phosphazine; and a make-up solvent of 90:10:0.1 MeOH:Water:Formic Acid which was introduced to the LC flow prior to the source to assist ionization. Melting points were determined on a Stanford Research Systems OptiMelt apparatus.

Synthesis of (*S*)-*N*-(2,5-Dimethylphenyl)-1-(quinolin-8-ylsulfonyl)pyrrolidine-2-carboxamide (5o)

To a mixture of *L*-proline (0.50 g, 4.34 mmol) in 10% K_2CO_3 (10 mL) and THF (10 mL) was added 8-quinolinesulfonyl chloride (1.98 g, 8.68 mmol), and the resulting mixture was stirred at 50 °C for 5 h. After cooling to room temperature, the reaction mixture was acidified with 3 N aqueous HCl to pH 2 and then extracted with EtOAc (3 × 30 mL). Separation and drying of the combined organic extracts ($MgSO_4$), followed by removal of solvent under reduced pressure afforded (*S*)-1-(quinolin-8-ylsulfonyl)pyrrolidine-2-carboxylic acid as a white solid (0.80 g, 60% yield) that did not require further purification and was used in the next step. To a solution of (*S*)-1-(quinolin-8-ylsulfonyl)pyrrolidine-2-

carboxylic acid (0.060 g, 0.20 mmol) in DMF (0.75 mL) was added 2,5- dimethylaniline (0.024 mL, 0.20 mmol), HATU (0.082 g, 0.22 mmol), and DIPEA (0.097 mL, 0.59 mmol). The reaction mixture was stirred for 2 h at room temperature, then diluted with CH_2Cl_2 (5 mL) and washed sequentially with aqueous 10% HCl (2 × 5 mL), saturated aqueous $NaHCO_3$ (2 × 5 mL), and water (2 × 5 mL). The separated organic extracts were dried ($MgSO_4$), and evaporated to give the crude product which was purified by silica gel flash column chromatography (2% MeOH in CH_2Cl_2) to afford (*S*)-*N*-(2,5-dimethylphenyl)-1-(quinolin-8-ylsulfonyl)pyrrolidine-2-carboxamide as a colorless oil (0.050 g, 62% yield). ^{1}H NMR (500 MHz; $CDCl_3$): δ (ppm) 9.54 (s, 1H), 8.88 (dd, *J* = 4.3 and 1.8 Hz, 1H), 8.62 (dd, *J* = 7.4 and 1.4 Hz, 1H), 8.28 (dd, *J* = 8.4 and 1.7 Hz, 1H), 8.11 (dd, *J* = 8.2 and 1.3 Hz, 1H), 7.69 (t, *J* = 7.8 Hz, 1H), 7.63 (s, 1H), 7.53 (dd, *J* = 8.3 and 4.3 Hz, 1H), 7.11 (d, *J* = 7.7 Hz, 1H), 6.93 (d, J = 7.6 Hz, 1H), 5.41 (dd, *J* = 7.9 and 2.0 Hz, 1H), 3.44-3.32 (m, 2H), 2.52-2.40 (m, 1H), 2.33 (s, 6H), 1.96-1.82 (m, 2H), 1.82-1.72 (m, 1H). ^{13}C NMR (126 MHz; $CDCl_3$): δ (ppm) 170.66, 151.58, 143.91, 137.12, 136.37, 135.88, 135.29, 134.92, 134.62, 130.45, 129.29, 127.65, 126.34, 125.84, 124.23, 122.51, 63.20, 49.29, 30.19, 24.98, 21.24, 17.65. LCMS purity (214 nm) =100%. HRMS: *m/z* calcd for $C_{22}H_{23}N_3O_3S$ ($M + H^+$) 410.1533, found 410.1532. Enantiomeric excess was determined by HPLC analysis: $[\alpha]_D^{25}$ −31.5 (c 0.0039 $CHCl_3$), > 99% ee.

Time of Addition Assay

HEp-2 cells were plated in 96 well black tissue culture plates at 10,000 cells per well in 100 μL and incubated 24 h at 37 °C, 5% CO_2. Test compounds were diluted in media to give a final concentration of 25 μM and added to plates in triplicate at −1, 0, 1, 2, 3, 5, 7, 21 and 24 h post-infection. Cells were infected with *h*RSV strain Long at an MOI of 3.0 at time point 0 and incubated for 6 days at 37 °C, 5% CO_2. Following a six day incubation period, the assay plates were equilibrated to room temperature for 30 min. An equal volume (100 μL) of Cell Titer-Glo reagent (Promega Inc.) was added to each well using a WellMate (Matrix, Hudson, NH) and the plates were incubated for an additional 10 min at room temperature. At the end of the incubation, luminescence was measured using a multi-label reader (PerkinElmer, Wellesley, MA) with an integration time of 0.1 s. Ribavirin was used as a control compound.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS USED

***h*RSV**	human respiratory syncytial virus
COPD	chronic obstructive pulmonary disease
GMP	guanosine monophosphate
GTP	guanosine triphosphate
IMPDH	inosine monophosphate dehydrogenase

RNA	ribonucleic acid
RNP	ribonucleoprotein
CPE	cytopathic effect
ATP	adenosine triphosphate
MLSMR	Molecular Libraries Small Molecule Repository
PBS	phosphate buffered saline
PI	post infection

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31. While compound **5b** shows inherent cellular toxicity, the observed levels of cellular viability for infected cells treated with **5b** did not appear so strongly affected such that a CPE was observed, albeit at the lower range of the three tested compounds in the series.

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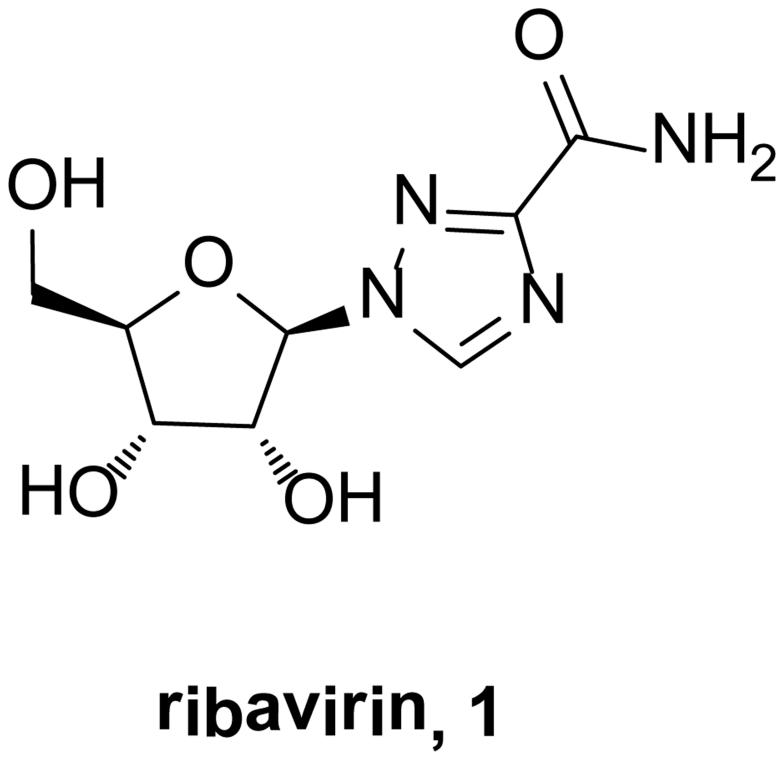


ribavirin, 1

Figure 1.
Structure of ribavirin **1**

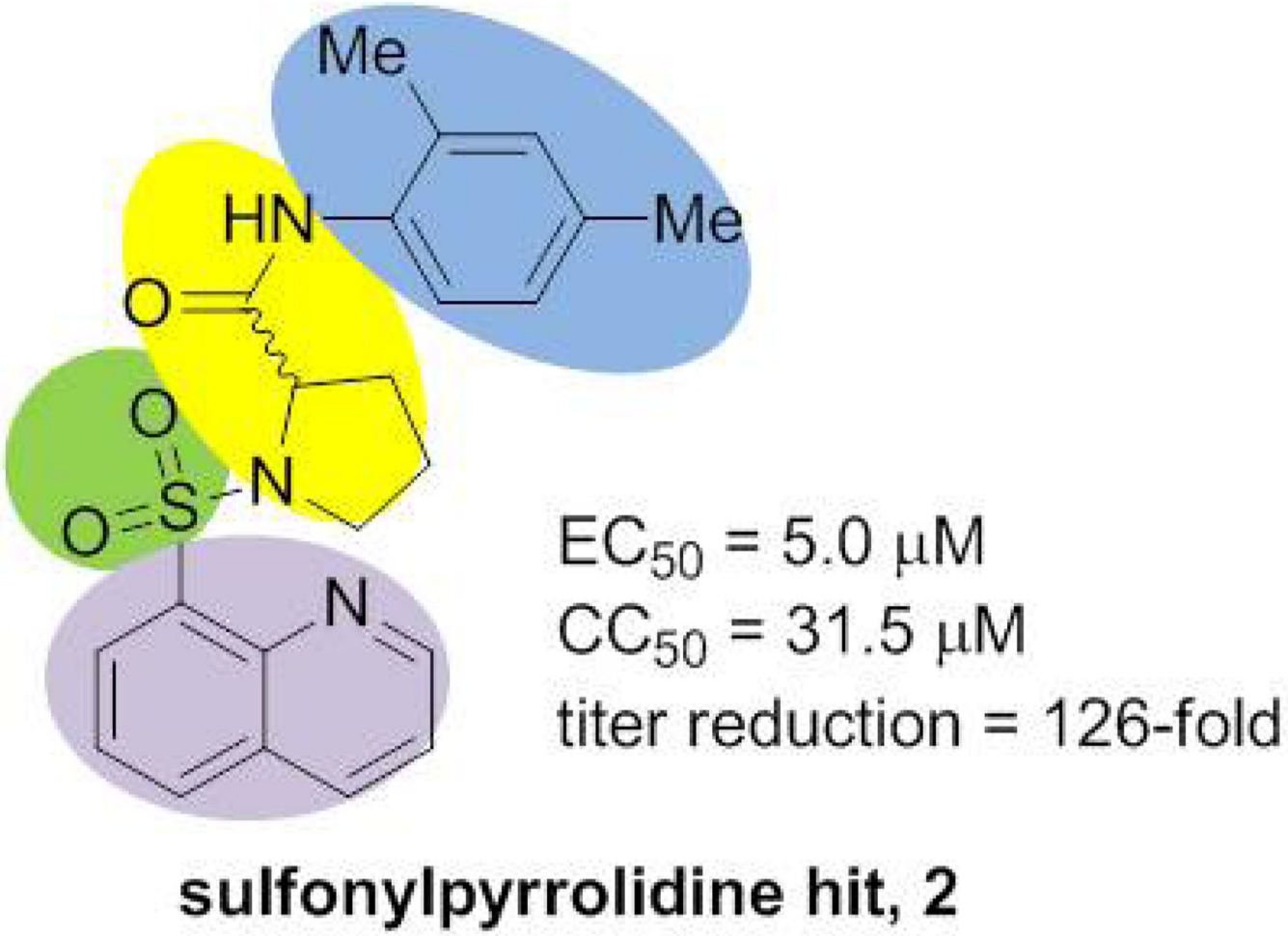


Figure 2.
Shaded regions of hit compound **2** optimized by structure-activity relationships.

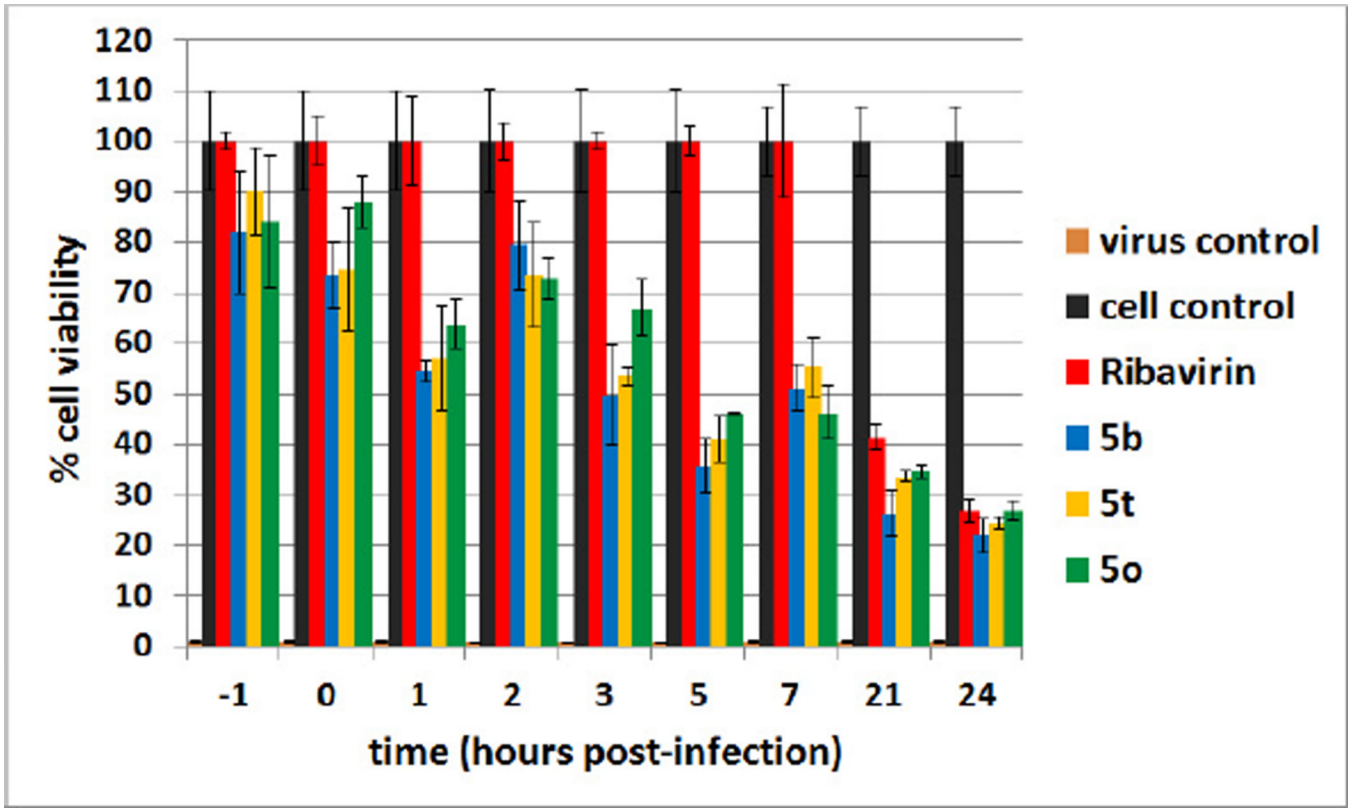
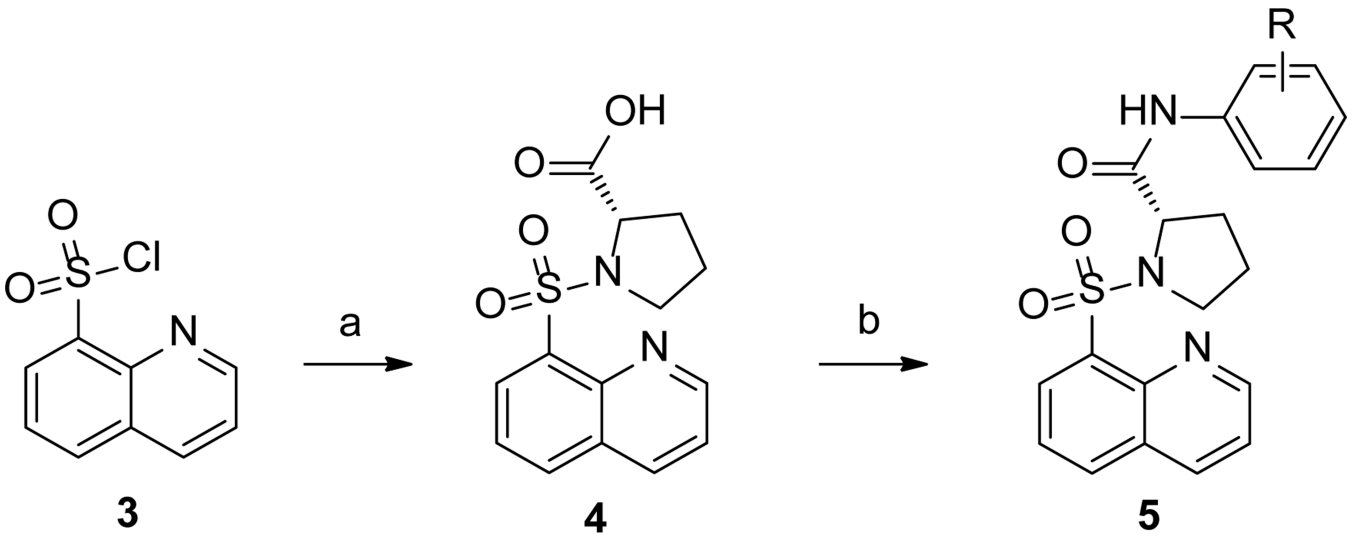


Figure 3.
Comparison of ribavirin with analogs **5b**, **5o** and **5t** in a time of addition assay.

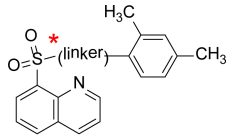
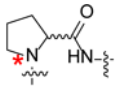
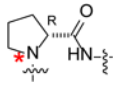
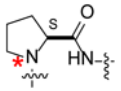
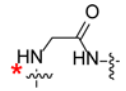
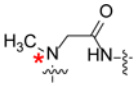
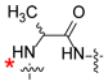


Scheme 1. Chemical Synthesis of Sulfonylpyrrolidine Analogs

Reagents and conditions: (a) *L*-proline, 10% aq. K_2CO_3, THF, 50 °C, 5 h, then 3N HCl; (b) DIPEA, HATU, substituted aniline, DMF, rt, 2 h.

Table 1

*h*RSV CPE Assay Potency, Cytotoxicity, Selectivity Index and Logarithmic Reduction in Viral Plaques for Analogs with Structural Variation in the Proline Linker Region of the Hit Sulfonylpyrrolidine Scaffold.

entry	compound	linker structure	CPE potency ± standard deviation (EC_{50}, μM)[a]	cytotoxicity ± standard deviation (CC_{50}, μM)[b]	selectivity index (CC_{50}/EC_{50})	Virus titer log reduction (plaque assay)[c]
		 linker structure				
		linker structure				
1	**1, ribavirin**	NA	28.4 ± 3.8	113.9 ± 38.5	4.0	2.5
2	**2**		5.0 ± 1.4	31.5 ± 5.7	6.3	2.1
3	**5a**		> 50.0	38.1 ± 2.3	0.8	NT
4	**5b**		2.7 ± 0.9	31.8 ± 7.7	11.8	1.0
5	**5c**		> 50.0	> 50.0	1.0	NT
6	**5d**		> 50.0	> 50.0	1.0	NT
7	**5e**		> 50.0	> 50.0	1.0	NT

[a] Data is an average of ≥ 3 experiments;

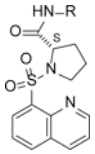
[b] Data is an average of ≥ 2 experiments;

[c] NT = not tested;

NA = not applicable; Data were analyzed using Microsoft® Excel 2010.

Table 2

*h*RSV CPE Assay Potency, Cytotoxicity, Selectivity Index and Logarithmic Reduction in Viral Plaques for Analogs with Structural Variation in the Aryl Amide Region of the Sulfonylpyrrolidine Scaffold.

entry	compound	 R	CPE potency ± standard deviation (EC_{50}, μM)[a]	cytotoxicity ± standard deviation (CC_{50}, μM)[b]	selectivity index (CC_{50}/EC_{50})	Virus titer log reduction (plaque assay)[c]
1	**1, ribavirin**	NA	28.4 ± 3.8	113.9 ± 38.5	3.6	2.5
2	**5b**	2,4-dimethylphenyl	2.7 ± 0.9	31.8 ± 7.7	13.1	1.0
3	**5f**	2-methylphenyl	23.8 ± 10.2	> 50.00	2.1	NT
4	**5g**	3-methylphenyl	> 50.0	25.5 ± 4.1	0.5	NT
5	**5h**	4-methylphenyl	> 50.0	31.7 ± 2.9	0.6	NT
6	**5i**	phenyl	> 50.0	35.6 ± 2.3	0.7	NT
7	**5j**	2-ethylphenyl	21.2 ± 5.1	> 50.0	2.6	NT
8	**5k**	2-*i*-propylphenyl	17.6 ± 0.9	29.9 ± 3.5	1.7	NT
9	**5l**	2,4-dimethyl-3-pyridine	> 50.0	> 50.0	1.0	NT
10	**5m**	3,5-dimethylphenyl	> 50.0	31.3 ± 3.5	0.6	NT
11	**5n**	2,4-dichlorophenyl	> 50.0	> 50.0	1.0	NT
12	**5o**	2,5-dimethylphenyl	2.3 ± 0.8	30.9 ± 1.1	13.4	2.0
13	**5p**	2,6-dimethylphenyl	> 50.0	> 50.00	1.0	NT
14	**5r**	2-methyl-5-chlorophenyl	2.2 ± 0.6	10.0 ± 1.3	4.3	NT
15	**5s**	2-methyl,5-trifluoromethylphenyl	> 50.0	4.8 ± 1.7	0.1	NT
16	**5t**	2-methyl,5-methoxyphenyl	4.2 ± 1.3	44.2 ± 5.7	10.4	1.0
17	**5u**	2-methyl,5-*i*-propylphenyl	> 50.0	9.7 ± 1.2	0.2	NT
18	**5v**	2-methyl,5-*t*-butylphenyl	> 50.0	6.5 ± 0.6	0.1	NT
19	**5w**	2-chloro,5-methylphenyl	> 50.0	> 50.0	1.0	NT
20	**5x**	2-methoxy,5-methylphenyl	> 50.0	> 50.0	1.0	NT
21	**5y**	(1-(*t*-butyl)-3-methyl-1H-pyrazol-5-yl	> 50.0	> 50.0	1.0	NT

[a] Data is an average of ≥ 3 experiments;

[b] Data is an average of ≥ 2 experiments;

[c] NT = not tested;

NA = not applicable; Data were analyzed using Microsoft® Excel 2010.